Phosphonium compounds as new and specific inhibitors of bovine serum amine oxidase

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TPP^+ (tetraphenylphosphonium ion) and its analogues were found to act as powerful competitive inhibitors of BSAO (bovine serum amine oxidase). The binding of this new class of inhibitors to BSAO was characterized by kinetic measurements. TPP^+ can bind to the BSAO active site by hydrophobic and by coulombian interactions. The binding probably occurs in the region of the 'cation-binding site'[Di Paolo, Scarpa, Corazza, Stevanato and Rigo (2002) Biophys. J. **83**, 2231–2239]. Under physiological conditions, the association constant of TPP^+ for this site is higher than 10^6 M^{-1}, the change of enthalpy being the main free-energy term controlling binding. Analysis of the relationships between substrate structure and extent of inhibition by TPP^+ reveals some new molecular features of the BSAO active site.

Key words: amine oxidases, competitive inhibitors, phosphonium compounds, structure–function relationships.

INTRODUCTION

Copper-containing AOs (amine oxidases) {amine:oxygen oxidoreductase (deaminating) [copper/TPQ (2,4,5-trihydroxyphenylalanine quinone)-containing]; EC 1.4.3.6} are a class of enzymes involved in the intra- and extra-cellular metabolism of various amines. These enzymes catalyse the two-electron oxidation of primary amines to the corresponding aldehydes with reduction of O_2 to H_2O_2, according to the following overall reaction [1]:

$$R\text{-}CH_2\text{-}NH_3^+ + O_2 + H_2O \xrightarrow{AO} R\text{-}CHO + NH_4^+ + H_2O_2 \qquad (1)$$

The nature of the covalently bound cofactor, TPQ, as well as the role of copper, and the steps of the catalytic mechanism, have been studied for some time and are well established [2,3], but, until now, only five AOs have been structurally characterized [4–6]. Copper-containing AOs are widespread in Nature, being found in organisms ranging from bacteria to plants and animals, but the definitive physiological role of these enzymes has not been well established. In fact, they may be involved in numerous metabolic pathways, since amines and their reaction products have been implicated in the processes of growth, differentiation and development of cells, apoptosis, wound healing, detoxification and also in glucose transport and lipolysis in adipocytes [7,8]. The assignment of functions to AOs is also complicated by the presence in some species of multiple AOs with different substrate specificity, depending on their location. An example is man (*Homo sapiens*), in which two genes plus a pseudogene encode the human semicarbazide-sensitive AOs that have been found in many tissues, for example in kidney, intestine, thymus gland, placenta, lung, cartilage, adipocytes, retina, plasma [7,8] and cardiovascular smooth-muscle cells {human VAP-1 (vascular adhesion protein-1) [9]}. Differences in the level of human AOs are linked to pathologies such as diabetes, atherosclerosis, congestive heart failure and tumours [8,10,11]. Research into new and selective inhibitors for AOs is an important goal, both to clarify the role played by these enzymes, particularly when they coexist in the same organism, and for their potential pharmacological applications. Studies of the mode of interaction between AOs and inhibitors are also essential in order to probe the molecular features of the AO active site, especially when their structure is not available.

To achieve these aims, a variety of compounds, such as carbonyl reagents or substrate analogues, have been used as AO inhibitors [7,12,13]. In particular, copper-containing AOs are usually inhibited by carbonyl reagents (semicarbazide, hydroxylamine, hydrazine and their derivatives), which react with the TPQ cofactor. Among these reagents, the hydrazides of pyrrol-1-ylphenylacetic acid are characterized by an inhibition constant (K_i) in the micromolar range in the case of BSAO (bovine serum AO) [13]. A variety of substrate analogues and poor substrates [14–16] were also used as inhibitors. However, few of them are characterized by an inhibition constant in the submicromolar range, such as *trans*-3-chloroallylamine, 3-5 ethoxy-4-aminomethylpyridine (B24) and *E*-2-phenyl-3-chloroallylamine (MDL72274) [16–19].

Here we report on new types of potent competitive inhibitors, namely phenylphosphonium salts, which are neither carbonyl reagents nor substrate analogues. They are a class of lipophilic molecules, important as chemotherapic agents [20,21], cationic biocides [22,23] and inhibitors of some enzymes such as protein kinase C [24] and HIV integrase [25]. They are also used as sensors of transmembrane potential [26]. Until now, no data on their effects on AO activity have been reported. Here, we report kinetic studies focused on characterizing the inhibitory effect of phenylphosphonium salts on BSAO. The results also provide new information about the active site of this mammalian enzyme.

EXPERIMENTAL

Materials

All chemicals were of the highest available quality and were used without further purification. In particular, the substrates used in

Abbreviations used: AO, amine oxidase; BSAO, bovine serum amine oxidase; BUA, 1-aminobutane; BZA, benzylamine; K_b, association constant; K_d, dissociation constant; K_i, inhibition constant; NONA, 1-aminononane; PSAO, pea seedling amine oxidase; PTP, propyltriphenylphosphonium; TPAs, tetraphenylarsonium; TPO, triphenylphosphine oxide; TPP(+), tetraphenylphosphonium (ion); TPQ, 2,4,5-trihydroxyphenylalanine quinone; SPD, spermidine; SPM, spermine; VAP-1, vascular adhesion protein-1.

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the present study were from Sigma–Aldrich–Fluka S.r.l. (Milan, Italy).

BSAO was purified as described by Vianello et al. [27]. The specific activity was 0.36 unit/mg of protein (one enzyme unit is defined as 1 μmol of substrate transformed/min). Soybean (*Glycine max*) seedling AO was purified by the method of Vianello et al. [28].

Enzyme activity measurements

Initial-rate measurements were carried out on a PerkinElmer Lambda-17 spectrophotometer, the peroxidase-coupled assay described by Di Paolo et al. [29] being followed. This method measures the oxidation rate of reduced cytochrome *c* by H_2O_2 in the presence of a mediator (*p*-hydroxyphenylacetic acid) and permits H_2O_2 generation rates as low as 60 $nM \cdot min^{-1}$ to be detected ($\Delta\varepsilon_{417} = 1.12 \times 10^5\ M^{-1} \cdot cm^{-1}$). The tested inhibitors do not affect this method, since the rate of ferrocytochrome oxidation by H_2O_2, injected into the assay solution in the absence of AO, was not affected by the presence of TPP^+ (tetraphenylphosphonium ion) and related compounds. All measurements were carried out at 25 °C, in air-equilibrated solutions – that is, under saturation conditions for molecular oxygen ($K_m^{O_2} \approx 10$–20 μM [30]). Under these conditions, the resulting kinetic parameters refer to the amines (see eqn 1). Ionic strength was controlled by the appropriate addition of NaCl.

Activity measurements, at various pH values, were performed in the appropriate buffer (25 mM) with NaCl (150 mM). Buffers were: Mes (pH 5.6–6.6), Mops (pH 6.6–7.3) and Hepes (pH 7.3–7.6). NaOH and HCl were used to adjust the pH. Activity measurements performed at overlapping pH values showed no significant influence of the type of buffer on k_{cat} and K_m values.

Standard assays, using a solution containing 0.5 mM SPM (spermine), 25 mM Hepes and 150 mM NaCl, at pH 7.20, were performed together with each set of rate measurements to check the specific activity of BSAO.

Multiple inhibitor experiments were carried out by incubating BSAO (1.6 μM) with reagents specific for the carbonyl group of TPQ in the presence and in the absence of TPP^+ (0.9 mM). After 30 min of incubation, the residual activity of the enzyme was monitored by dilution (1:360) in the assay solution containing 2 mM SPM as substrate.

Concentrations of purified enzymes were determined by the method of Bradford [31], assuming a molecular mass for the enzyme of 180 kDa.

Data analysis

The steady-state kinetic parameters of BSAO (k_{cat}, K_m and k_{cat}/K_m) were calculated by fitting the experimental data to the Michaelis–Menten equation using a non-linear regression analysis program. The parameter k_{cat} is the number of molecules of substrate transformed/s per catalytic centre (two per BSAO molecule). In some cases, k_{cat}/K_m was calculated directly from experiments carried out at substrate concentrations ([S]) much lower than the K_m, according to the Michaelis–Menten equation. In the case of competitive inhibition, the inhibition constants, which correspond to the dissociation constants of the enzyme–inhibitor complex (K_d) were determined by plotting the values of the slopes obtained by the Lineweaver–Burk method versus inhibitor concentration according to the Dixon equation:

$$K_{m,app}/K_{m,0} = (1 + [I]/K_d)$$

where $K_{m,app}$ and $K_{m,0}$ are the Michaelis–Menten constant calculated in the presence and absence respectively of the inhibitor [32]).

Table 1 Residual SPM oxidase activity of BSAO in presence of a 1 mM concentration of various onium compounds

All measurements were carried out in 25 mM Hepes/150 mM NaCl, at pH 7.20 and 25 °C, using 4 μM SPM ([SPM] $\ll K_m = 30$ μM).

Compound	Residual activity (k_{cat}/K_m) (%)*
Tetramethylphosphonium chloride	83
Tetrabutylphosphonium chloride	25
Methyltriphenylphosphonium chloride	1
Ethyltriphenylphosphonium chloride	1
PTP chloride	0.5
TPP^+ chloride	0.5
TPAs chloride	2
Ammonium chloride	96
Tetramethylammonium chloride	88
Tetraethylammonium chloride	75
Tetrabutylammonium bromide	56

* 100 % of $(k_{cat}/K_m)_{SPM} = 3.7 \times 10^4\ M^{-1} \cdot s^{-1}$.

The pK_a values of the amino acid residues involved in the binding of TPP^+ were calculated by fitting the association constants of TPP^+ for BSAO [(K_b)] to the following Hill equation (modified by Markley [33]):

$$K_b = (K_b)_a \left[1 + 10^{h(pK_a - pH)}\right]^{-1} + (K_b)_c \left\{1 - \left[1 + 10^{h(pK_a - pH)}\right]^{-1}\right\} \quad (2)$$

where $(K_b)_a$ and $(K_b)_c$ are the values of K_b measured when the inhibitor binding site(s) are deprotonated and protonated respectively, and h is the Hill coefficient. This coefficient represents the number of protons involved in the TPP^+ binding site or the degree of co-operativity on TPP^+ binding.

Data were fitted to the appropriate equation by using the least-squares method and the Sigma Plot 5.0 program (Jandel Scientific, San Rafael, CA, U.S.A.). The value of the parameter obtained from the best fit and its S.E.M. are reported. Unless otherwise mentioned, the correlation coefficient for linear regression was 0.98 or better. All experiments were performed in triplicate.

The Dynafit program (BioKin Ltd, WA, U.S.A.; http://www.biokin.com) was used, when necessary, to perform the global fits of the data to assess the best fit to various inhibition mechanisms (model discrimination analysis) and to calculate the inhibition constants (for details, see the Results and discussion section).

RESULTS AND DISCUSSION

Effects of some onium compounds on SPM oxidase activity

The effects on the activity of BSAO of various positively charged onium compounds were tested, using SPM as substrate. Strong inhibition of activity was measured at [SPM] $\ll K_m$, but no significant effect was observed at [SPM] $\gg K_m$. No dependence of inhibition by onium compounds on time was observed. Table 1 shows the residual catalytic efficiency (k_{cat}/K_m) measured in the presence of a 1 mM concentratrion of the various onium compounds at [SPM] $\ll K_m$. It appears that the inhibitory effect increases with the length of the aliphatic chains of the inhibitor, and that the lowest residual activity is observed when phenyl groups are substituents. One explanation of this phenomenon may be the lipophilic nature of these compounds, as well as the higher sterical hindrance of the phenyl groups.

To study the mechanism of inhibition of these compounds, TPP, tetraphenylarsonium (TPAs) and PTP (propyltriphenylphosphonium) chloride were chosen as representative molecules. The K_m and k_{cat} values for SPM were calculated, in the absence

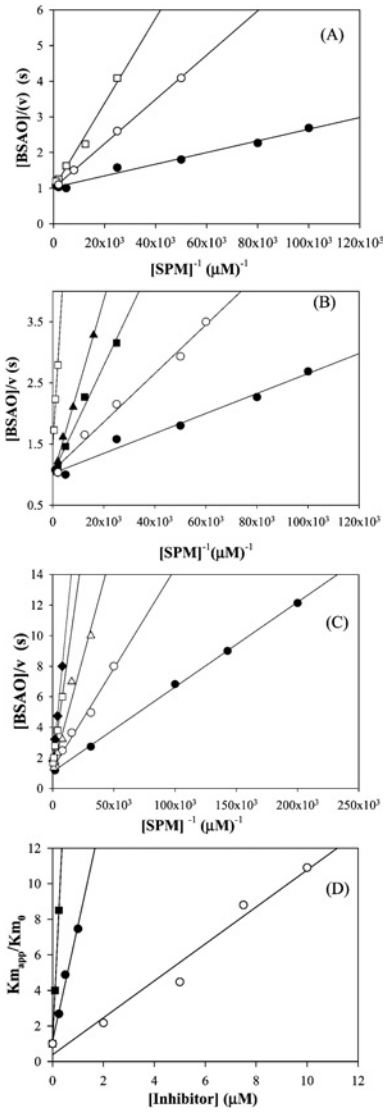


Figure 1 Phosphonium compounds as competitive inhibitors of the SPM oxidase activity of BSAO

Lineweaver–Burk plots for the inhibition of BSAO by various inhibitors (**A**–**C**) are shown. All the measurements were performed in 25 mM Hepes/150 mM NaCl at pH 7.20 and 25 °C. The following concentrations for the various compounds were used: (**A**) PTP chloride at 0 (●), 0.1 (○) and 0.25 (□) μM; (**B**) TPP$^+$ chloride at 0 (●), 0.25 (○), 0.5 (■), 1 (▲) and 10 (□) μM; and (**C**) TPAs chloride at 0 (●), 2 (○), 5 (△), 7.5 (□) and 10 (◆) μM. (**D**) Dixon plots obtained for the various inhibitors from the K_m values calculated from the double-reciprocal plots (**A**–**C**): 0, TPAs chloride; ●, TPP$^+$ chloride; ■, PTP chloride.

or presence of inhibitor at various concentrations, by fitting the experimental data to the Michaelis–Menten equation. In all cases, only K_m depended on inhibitor concentration, and no significant change in k_{cat} was observed ($k_{cat,SPM} = 1.10 \pm 0.18\ s^{-1}$), as shown in Figures 1(A)–1(C). These results indicate that these compounds behave as competitive inhibitors when SPM is used as substrate. Figure 1(D) shows the dependence of the $K_{m,app}/K_{m,0}$ ratio on inhibitor concentration. According to the classical Dixon equation, the following dissociation constants (K_d) were calculated for various BSAO–inhibitor complexes when SPM was used as substrate: $K_{d,PTP} = 30$ nM, $K_{d,TPP^+} = 150$ nM and $K_{d,TPAs} = 960$ nM. These K_d values, which are in the submicromolar range, clearly indicate that PTP, TPP$^+$ and TPAs are a new and strong group of competitive inhibitors. AO inhibitors with similar inhibition constant values were reported only for some substrate analogues [13,17,18,34,35].

Table 2 Substrate structure and TPP inhibition

All measurements were carried out at [S] $\ll K_m$, ($K_{m,SPD} = 300\ \mu$M; $K_{m,NONA} = 15\ \mu$M; $K_{m,BZA} = 1.5$ mM; $K_{m,BUA} = 2.5$ mM). For other experimental conditions, see Table 1.

	Residual activity (k_{cat}/K_m) (%)*		K_d† (μM)	
Substrate	[TPP] = 0.5 μM	[TPP] = 1 mM	By Dynafit program	By Dixon method
SPM	20.9 ± 2.1	≈0 ± 0.5	0.15 ± 0.03	0.15 ± 0.04
SPD	25.3 ± 3.2	≈0 ± 0.5	0.15 ± 0.03	0.17 ± 0.04
NONA	44.4 ± 2.3	2.0 ± 1.5	0.40 ± 0.06	0.38 ± 0.08
BZA	38.6 ± 2.2	18.2 ± 2.1	0.27 ± 0.08	0.61 ± 0.11‡
BUA	45.9 ± 5.0	22.1 ± 2.1	0.37 ± 0.13	0.49 ± 0.07‡

* The following reference values (100 %) have been calculated under our experimental conditions: $(k_{cat}/K_m)_{SPM} = 3.7 \times 10^4\ M^{-1} \cdot s^{-1}$, $(k_{cat}/K_m)_{SPD} = 3 \times 10^3\ M^{-1} \cdot s^{-1}$, $(k_{cat}/K_m)_{NONA} = 2.5 \times 10^4\ M^{-1} \cdot s^{-1}$, $(k_{cat}/K_m)_{BZA} = 4.0 \times 10^2\ M^{-1} \cdot s^{-1}$, $(k_{cat}/K_m)_{BUA} = 2.5 \times 10^2\ M^{-1} \cdot s^{-1}$. The experimental substrate concentrations were: [SPM] = 4 μM, [SPD] = 25 μM, [NONA] = 2.5 μM, [BZA] = 0.2 mM and [BUA] = 0.2 mM.

† K_d calculated according to the Dynafit program and the Dixon equation.

‡ K_d calculated according to the Dixon equation from the data of Figure 2 at a TPP concentration lower than 1 μM, that is, from the linear part of the curve.

TPP$^+$ inhibition and substrate structure

The catalytic efficiency of BSAO towards various substrates was measured in the presence of 0.5 μM and 1 mM TPP$^+$ (Table 2). SPM, SPD (spermidine), NONA (1-aminononane), BUA (1-aminobutane) and BZA (benzylamine) were chosen as substrates owing to the different types of interaction they show with the BSAO active site: docking of SPM, SPD, BUA and BZA appears to be controlled by electrostatic interactions, and that of NONA by hydrophobic interactions with a different region of the active site [36]. Table 2 shows that, at submicromolar concentrations, TPP$^+$ behaves as a strong inhibitor of all tested substrates (residual activity not higher than 20–50 %, independently of the enzyme concentration). However, the significant residual activity measured in the presence of 1 mM TPP$^+$ in the case of BUA and BZA does not fit purely competitive behaviour. In fact, in this case, activity near zero was expected, as was found with SPM, SPD and NONA. The significant residual activity measured in the presence of 1 mM TPP$^+$ in the case of BUA and BZA was not due to a decrease in the concentration of TPP$^+$ in the presence of the various substrates, since no precipitate or aggregate (no change in the Fourier-transform IR spectra) was observed, even at 0.1 M TPP$^+$ and BZA or BUA. For a better insight into TPP$^+$ inhibition, the K_m and k_{cat} values for the various substrates were measured in the presence of various concentrations of TPP$^+$. In all cases, only the K_m values were found to be significantly dependent on TPP^{++} concentration (the following mean values of k_{cat} were measured for the various substrates: $k_{cat,SPM} = 1.10 \pm 0.20\ s^{-1}$; $k_{cat,SPD} = 0.92 \pm 0.11\ s^{-1}$; $k_{cat,NONA} = 0.37 \pm 0.04\ s^{-1}$; $k_{cat,BUA} = 0.74 \pm 0.10\ s^{-1}$; and $k_{cat,BZA} = 0.63 \pm 0.12\ s^{-1}$). However, as shown in Figure 2, whereas in the case of SPM, SPD and NONA, $K_{m,app}/K_{m,0}$ ratios increase linearly with TPP^{++} concentration, with BZA and BUA a non-linear increase in the $K_{m,app}/K_{m,0}$ ratio was observed, the asymptotic value also being maintained at 1 mM TPP$^+$ (results not shown).

The analysis of these results suggests that: (i) substrates with a shorter tail (BZA, BUA), unlike those with a longer tail (SPM, SPD and NONA) may react even in the presence of the inhibitor in the active site, that is, the ESI (enzyme–substrate–inhibitor complex) may give final products of reaction (P) with the

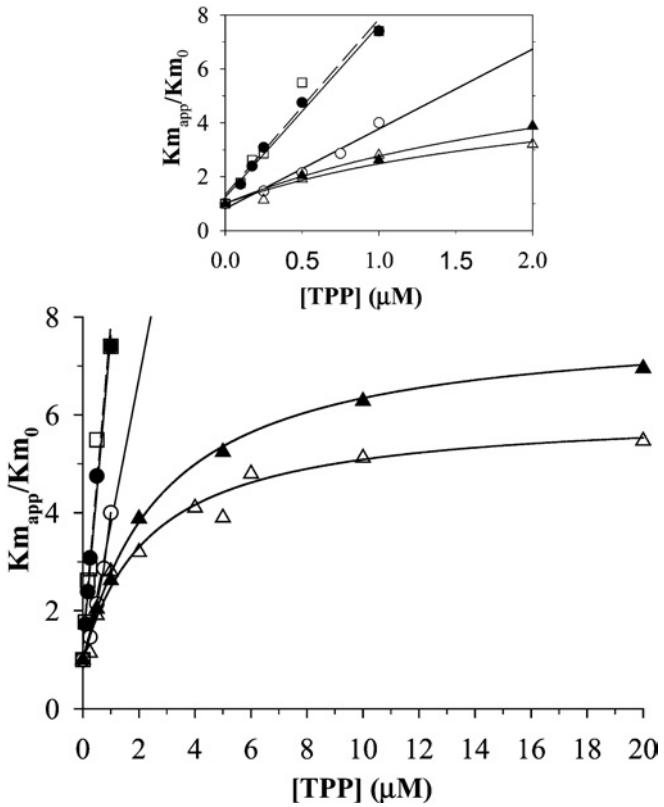
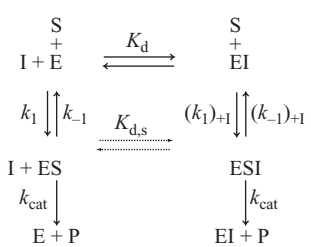


Figure 2 Inhibitory effect of TPP$^+$ on BSAO activity using various substrates

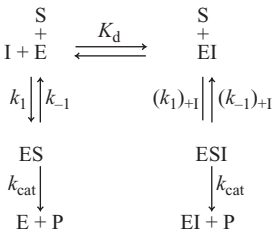
A plot of the $K_{m,app}/K_{m,0}$ ratio against the TPP$^+$ concentration is shown. All measurements were performed in 25 mM Hepes/150 mM NaCl, at pH 7.20 and 25 °C. The continuous curve was obtained by fitting experimental data to the Dixon equation for SPM, SPD and NONA, and to eqn (4) in the case of BUA and BZA. The following substrates were used: □, SPD; ●, SPM; ○, NONA; ▲, BZA; and △, BUA. Inset: magnification of the Figure at low TPP$^+$ concentrations.

same catalytic constant as the ES (enzyme–substrate complex); (ii) shorter substrate may enter the active site even in the presence of TPP$^+$, along another pathway, although with greater difficulty or, alternatively, the inhibitor may bind to the preformed ES complex with a different dissociation constant ($K_{d,s}$) compared with that for the free enzyme (K_d) in accordance with the partial inhibition mechanism, or all these possibilities may occur. Starting from the general scheme developed by Leidler and Bunting [37], the following total scheme of reaction, which represents all the possibilities listed above, can be drawn in the case of BSAO–TPP$^+$ and using BZA or BUA as substrate (Scheme 1a):



Scheme 1a

The Dynafit program was used to verify the inhibition mechanism for the various substrates. The experimental data (velocities at different substrate concentrations versus different inhibitor concentrations) were fitted to inhibition models and model discrimination analyses were performed. The following inhibition models were tested: competitive (I can bind only to the free E in place of S), uncompetitive (I binds only to ES in place of S to give EI and free S), partial competitive (I may bind both to the free E and the ES complex and both ES and ESI can give the final products), mixed (I can bind both to E and ES, but only ES gives the final products of reaction), 'total model' (reported in Scheme 1a) and the one we termed 'partial-original' (where S may bind to EI without substituting I and both ES and ESI may give the final product) according to Scheme 1(b):



Scheme 1b

In the case of SPM, SPD and NONA, the optimal fit was obtained with the competitive model, confirming the behaviour found using the Dixon equation. In the case of BUA and BZA, the model discrimination analysis, on the basis of the best fit of the various models, 'accepted' only the 'partial-original' mechanism of inhibition. The competitive, partial competitive, mixed and uncompetitive models were rejected by the Dynafit program owing to the poor fits of the experimental data. The 'total model' reported in Scheme 1(a) has not been accepted by the program, since it appears less correct (Occam's Razor) than the 'partial-original'. These results confirm that, in the presence of TPP$^+$, the shorter substrates enter the BSAO active site along another pathway, though with more difficulty. K_d values calculated on the basis of the optimal fits are reported in Table 2. In this Table (last column), the K_d values calculated according to the Dixon equation are also reported. From the comparison of the data of the third and the last columns of Table 2, it clearly appears that a good agreement of the K_d values was found only in the case of SPM, SPD and NONA, that is, in the case of the competitive behaviour of TPP$^+$. On the basis of the analyses carried out using the Dynafit program, the equation of the reaction rate, v, for the mechanism of Scheme 1(b) has been written introducing steady-state conditions for all intermediates and assuming very fast equilibrium conditions for the E–I system (horizontal reactions) with respect to E–S reactions ('vertical' reactions" [37]):

$$v = k_{cat}[E]_o[S]/(K_{m,app} + [S]) \quad (3)$$

where $K_{m,app} = K_{m,0}(1 + [I]/K_d)/[1 + (K_{m,0}/K_{m+I})[I]/K_d]$. $K_{m,0}$ and $K_{m+I} = (k_{-1,+I} + k_{cat})/k_{1,+I}$ are the Michaelis–Menten constants for the BZA (or BUA) of free enzyme and of the BSAO–TPP$^+$ complex respectively. In the presence of TPP$^+$, the $K_{m,app}/K_{m,0}$ ratio (shown in Figure 2) is given by:

$$K_{m,app}/K_{m,0} = [(1 + [I]/K_d)]/\{1 + (K_{m,0}/K_{m,+I})[I]/K_d\} \quad (4)$$

The values of the parameters, $K_{m,+I} = 10.9 \pm 1.1$ mM in the case of BZA and $K_{m,+I} = 16.6 \pm 6.1$ mM in the case of BUA, were calculated from the above reported analysis performed using the Dynafit program.

The best fit obtained with the partial-original mechanism indicates that TPP$^+$ competes for the 'main entrance' towards the TPQ cofactor with small substrates, which, unlike the longer

ones, have an alternative route to TPQ, although less favoured. In other words: (i) BZA or other small substrates can reach the TPQ cofactor by two different docking pathways: the 'preferred' favourite pathway (blocked by TPP^+), which is the same as that followed by SPM, SPD and NONA, and a second pathway (not inhibited by TPP^+), that cannot be used by longer substrates. This second pathway explains the high residual activity measured at 1 mM TPP^+; (ii) TPP^+ interacts with an active site area relatively far from the TPQ cofactor. This area is involved in the binding of the tail of substrates longer than 5–6 Å (0.5–0.6 nm) (SPM, SPD and NONA) and should include the region we hypothesized to accommodate cations such as Cs^+ and Ca^{2+} [38]; (iii) the slight but significant higher association constant ($K_b = K_d^{-1}$) values for TPP^+ obtained using highly charged substrates such as SPM and SPD ($K_b \approx 6.2 \times 10^6\ M^{-1}$) in comparison with those obtained using NONA, BUA and BZA ($K_b \approx 2.9 \times 10^6\ M^{-1}$) suggest that hydrophobic or partially hydrophobic substrates competing with TPP^+ for the active site displace the inhibitor more easily than highly charged substrates. This behaviour could be due to the presence of a hydrophobic region in the active site, which interacts very strongly with molecules such as NONA or TPP^+ (phenyl rings). A different behaviour of an inhibitor according to the substrate employed has already been hypothesized for some enzymes (angiotensin-converting enzyme, acetylcholinesterase and human cytochrome P450 1A2) [39–41].

Characterization of BSAO binding site for TPP^+

(A) Electrostatic interactions

The dependence of the association constant of the BSAO–TPP^+ complex on ionic strength (I) was studied at pH 7.2, using SPD as substrate. Following the Debye–Huckel theory [42], the data were fitted to the following equation:

$$\log K_b = \log K_{b,0} + 2C\,Z_a Z_b I^{1/2} \quad (5)$$

where Z_a and Z_b are the charges of the species involved in the formation of the enzyme–inhibitor complex, $K_{b,0}$ is the value of the association constant at $I = 0$ and $C = 0.497\ M^{1/2}$ in water at 25 °C.

According to eqn (5), the plot of $\log K_b$ versus $I^{1/2}$ was found to be linear ($r > 0.98$) in the I range 22–162 mM (results not shown). From the slope, a value of $Z_a Z_b = -1.5 \pm 0.2$ was calculated. This result indicates that at least one negative charge in the active site is involved in the ionic interaction with the phosphonium molecule.

To verify the involvement of the electrostatic interactions between TPP^+ and BSAO, TPO (triphenylphosphine oxide), a compound similar to TPP^+, but lacking the positive charge, was chosen as inhibitor. Using SPD as substrate, TPO behaves as a competitive inhibitor less powerful than TPP^+ ($K_{d,TPO} = 7.0 \pm 1.2\ \mu M$ in comparison with $K_{d,TPP^+} = 0.15\ \mu M$). However, in contrast with TPP^+, the K_d value for TPO is not affected by ionic strength in the I range 22–162 mM (results not shown). These results confirm the role of the positive charge of TPP^+ (R-P^+ moiety, where R are the phenyl rings of TPP^+) in the interaction with the BSAO active site.

(B) Effect of pH on K_b of BSAO and TPP^+

The K_b dependence of the BSAO–TPP^+ system on pH was studied using SPD as substrate. K_b values were calculated according to the Dixon equation, measuring the experimental $K_{m,app}/K_{m,0}$ ratio over the pH range 5.6–7.6. A fixed TPP^+ concentration of 0.2 μM was used. In the explored pH range, no effect of TPP^+ on k_{cat} values was found.

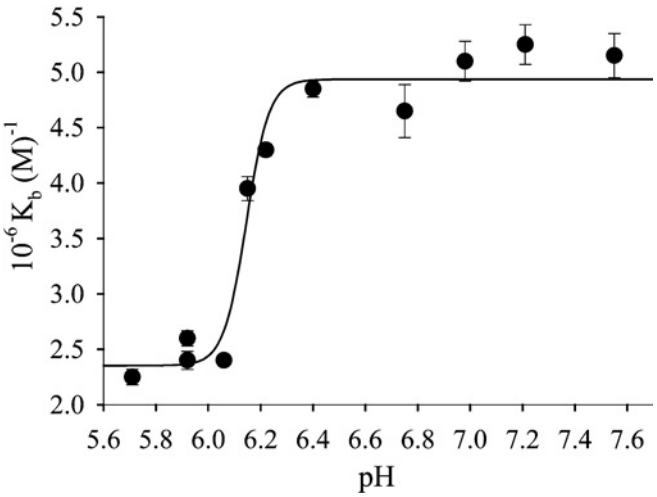


Figure 3 Dependence of BSAO–TPP^+ K_b on pH

K_b values calculated at various pH values and at 25 °C are shown. SPD was used as substrate. The continuous line was obtained by non-linear least-squares fit of experimental data to eqn (2).

Figure 3 shows the dependence of K_b values on pH. The K_b value increased from $(2.3 \pm 0.2) \times 10^6\ M^{-1}$–$(5.0 \pm 0.4) \times 10^6\ M^{-1}$ when the pH value was increased from 6.0 to 6.4. This behaviour suggests the presence of one or more residues with a $pK_a = 6.15 \pm 0.02$ (calculated using eqn 2; see the Experimental section) that, in the deprotonated state, favour, but do not control, the binding of TPP^+. In other words, TPP^+ may bind with more difficulty, even when these residues are fully protonated. From the dependence of K_b on pH it appears that the pK_a of 6.15 matches very well that previously found for residues (probably aspartic acid/glutamic acid) located at about 10 Å (1 nm) from the TPQ cofactor [36]. These residues, as previously reported [36], are also involved in the electrostatic binding of the amino group present in position 10 of SPD and SPM and in the binding of cations such as Cs^+ and Ca^{2+} [38], that act as competitive inhibitors only for long and highly charged substrates (SPM and SPD). In the case of TPP^+, in addition to the electrostatic interactions between the phosphonium ion and the negative charges of these residues, other forces, such as hydrophobic ones, appear involved in the binding of this large molecule to BSAO.

(C) Effect of temperature on binding of TPP^+ to BSAO

The dependence of association constant for TPP^+–BSAO complex on temperature was studied, using SPD as substrate. Experimental results show a decrease in the K_b value as the temperature increased from 27 to 52 °C. In accordance with the Arrhenius law, the plot of $\ln K_b$ versus T^{-1} was linear ($r = 0.997$) (Figure 4). A ΔH° value of $-35.28\ kJ\ mol^{-1}$ and a ΔS° value of $5.84\ J \cdot mol^{-1} \cdot K^{-1}$ were calculated for the binding of TPP^+ to BSAO, the data being fitted to the vant'Hoff equation [43]:

$$\ln K_b = -\Delta H^\circ/(RT) + \Delta S^\circ/R$$

The calculated ΔH° and ΔS° values show that both enthalpic and entropic factors favour the formation of the complex, the enthalpic term being dominant.

(D) Effect of TPP^+ on the binding of carbonyl reagents

Hydrazides, which are specific reagents of the carbonyl group, react with the TPQ cofactor, thus inhibiting the enzyme activity [44]. Two different hydrazides were used: acetic hydrazide, a

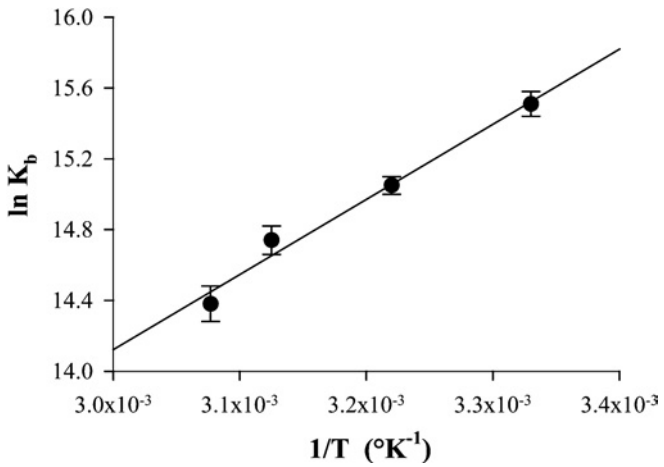


Figure 4 Arrhenius plot of the BSAO–TPP$^+$ association constant versus temperature

Experiments were carried out in 25 mM Hepes/150 mM NaCl, at pH 7.2, using SPD as substrate.

Table 3 Effect of TPP on the inhibition of BSAO by hydrazides

The incubation solution was 25 mM Hepes/150 mM NaCl, at pH 7.20 and 25 °C containing 1.6 μM BSAO. When indicated, 0.9 mM TPP was present. The assay solution was 25 mM Hepes/100 mM NaCl, pH 7.2, containing 2 mM SPM as substrate.

Hydrazide used and its concentration	Residual activity after the incubation time (%)*	
	− TPP†	+ TPP‡
Acetic hydrazide (38 μM)	30	38
Phenylacetic hydrazide (30 μM)	33	97

* The residual activity was monitored after diluting the incubated enzyme (1:360) in the assay solution; the activity of BSAO in the presence of TPP alone (2.5 μM final concn.) in the assay solution was taken as 100 %.

† 2.5 μM TPP was added to the assay solution to perform these activity measurements in the presence of the same concentration of TPP as in the assay solution of the samples incubated in the presence of TPP.

‡ 0.9 mM TPP was present in the incubation solution.

small and relatively non-hydrophobic molecule, and phenylacetic hydrazide, a large molecule characterized by the presence of a phenyl ring that could interact with the hydrophobic pocket of the BSAO active site [44]. The inhibition of BSAO was monitored after incubation of the enzyme for 30 min with these hydrazides in both the presence and absence of TPP$^+$. When TPP$^+$ was present, the hydrazide was added after TPP$^+$. The results obtained, reported in Table 3, clearly show that, in the case of phenylacetic hydrazide, the presence of TPP$^+$ affords practically total protection. Conversely the relatively smaller and non-hydrophobic acetic hydrazide is partially hindered by TPP$^+$ from reacting with the carbonyl group of TPQ. This behaviour strongly supports the notion that TPP$^+$ interacts with a hydrophobic region of the BSAO active site involved in the binding of hydrophobic molecules (inhibitors and substrates) and also support the possibility that small substrate or of inhibitor molecules can reach the active site (TPQ) whilst still in the presence of TPP$^+$.

Effect of TPP$^+$ on various AOs

To verify the specificity of TPP$^+$ inhibition, we tested and compared the effect of this compound on various AOs. Table 4 shows the residual activity of various AOs measured in the presence of 1 mM TPP$^+$, at [S] $\ll K_m$. Each enzyme was tested using a substrate for which the AO shows high catalytic efficiency.

Table 4 Effect of TPP on the activity of various AOs

Experiments were carried out in 25 mM Hepes/150 mM NaCl/1 mM TPP at pH 7.20 and 25 °C. For human AOs, the activity measurements were carried out at 37 °C as described by Holt et al. [49]. All measurements were carried out at [S] $\ll K_m$ for various enzyme/substrate systems.

Source of AO	Substrate	Residual activity (k_{cat}/K_m) (%)
Bovine serum*	SPM	0
Pig kidney	Cadaverine	55
Soybean seedling	Cadaverine	100
Human platelets*	BZA, NONA	100
Human serum*	Cadaverine	33

* Bovine serum, human serum and platelets were used without further purification.

Table 4 clearly shows that TPP$^+$ behaves as a potent and specific inhibitor only of BSAO, even when bovine plasma is used without any purification. In fact, in the case of other mammalian copper AOs (pig kidney AO, human serum AO), the high residual activity indicates a K_d in the millimolar range, while in the case of copper AOs from plants (purified soybean seedling AO) and of FAD-dependent AOs (in human platelets), no effect on activity was observed under our experimental conditions.

Conclusions

Phosphonium and arsonium ions were found to be a new class of onium compounds that are strong competitive inhibitors of BSAO.

The inhibitory effect of these compounds depends on the presence of a positive charge and of a relatively bulky hydrophobic moiety. The positive charge helps the electrostatic attraction of the inhibitors by negative charges of the active site as found for some substrates, and the hydrophobic moiety (phenyl rings) favours the binding of the inhibitor to the hydrophobic area of the active site, the presence of which has been suggested in various AOs, including BSAO [36,45,46].

In particular, the results we have obtained suggest that: (i) the TPP$^+$ positive moiety interacts electrostatically with the 'cation-binding site' (p$K_a \approx 6.2$), that is, with the region involved in the electrostatic interaction with the positive amino group of the tail of long polyamines and located about 10 Å (1 nm) from the TPQ cofactor. Here, at physiological pH, negative charges of deprotonated acid residues (probably aspartic acid/glutamic acid) are present; (ii) the aromatic moiety of TPP$^+$ interacts with a hydrophobic area near the cation-binding site at some distance from the TPQ; (iii) the presence of TPP$^+$ in the active site prevents docking of substrates and inhibitors with long and positively charged tails or bulky hydrophobic substituents; (iv) the presence of TPP$^+$ hinders, but does not prevent, docking of 'small' substrates such as BZA and BUA or small carbonyl reagents, which can reach the TPQ cofactor using a pathway less favoured with respect to the preferred one.

These findings seem to indicate that the access to BSAO active site is similar to that found for the lysyl oxidase of the yeast *Pichia pastoris* [5], namely that there should be a large cavity, quite open towards the surface of the protein and the solvent, and not the narrow channel found in other known copper AO structures [5,6]. The binding of large cations, such as phosphonium or arsonium compounds, to the cation-binding site of the BSAO active site, partially closes this large entrance. Consequently these onium compounds block the preferred pathway of substrate towards TPQ and fully inhibits the reaction of long-tailed substrates. Conversely, small substrates, such as BUA and BZA, in the presence

of these large cations, can still reach the cofactor by a less favoured route, and then react.

In addition to this new structural information, our findings could be of pharmacological interest, since the inhibitory effect of phosphonium compounds can be modulated according to the nature and to the sterical hindrance of the substituents, and human VAP-1, characterized by a sequence similarity with BSAO higher than 80 % [9], is involved in various pathogenic events.

Finally, possible effects of TPP^+ on VAP-1 and other human copper AOs should be considered when this and related compounds are used for clinical purposes as cationic biocides, antagonists in the vasorelaxant response to levcromakalim [47], chemotherapeutic agents [21] and molecular probes for imaging tumours [48]). In fact, in these cases, TPP^+ is used in a concentration range equal to, or higher than, that at which we observed inhibition of BSAO.

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REFERENCES

1 Knowles, P. F. and Dooley, D. M. (1994) Amine Oxidases. In Metal Ions in Biological Systems (Sigel, H. and Sigel, A., eds.), vol. 30, pp 361–403, Dekker, New York

2 Padiglia, A., Medda, R., Bellelli, A., Agostinelli, E., Morpurgo, L., Mondovì, B., Finazzi-Agrò, A. and Floris, G. (2001) The reductive and oxidative half-reactions and the role of copper ions in plant and mammalian copper-amine oxidases. Eur. J. Inorg. Chem. **1**, 35–42

3 Mure, M., Mills, S. A. and Klinman, J. P. (2002) Catalytic mechanism of the topaquinone containing copper amine oxidases. Biochemistry **41**, 9269–9278

4 McGuirl, M. A. and Dooley, D. M. (1999) Copper-containing oxidases. Curr. Opin. Chem. Biol. **3**, 138–144

5 Duff, A. P., Cohen, A. E., Ellis, P. J., Kuchar, J. A., Langley, D. B., Shepard, E. M., Dooley, D. M., Freeman, H. C. and Guss, J. M. (2003) The crystal structure of *Pichia pastoris* lysyl oxidase. Biochemistry **42**, 15148–15157

6 Wilce, M. J. C., Dooley, D. M., Freeman, H. C., Guss, J. M., Matsunami, H., McIntire, W. S., Ruggiero, C. E., Tanizawa, K. and Yamaguci, H. (1997) Structures of the copper-containing amine oxidase from *Arthrobacter globiformis* in the holo and apo forms: implications for the biogenesis of topaquinone. Biochemistry **36**, 16116–16133

7 Lyles, G. A. (1996) Mammalian plasma and tissue-bound semicarbazide-sensitive amine oxidase: Biochemical, pharmacological and toxicological aspects. Int. J. Biochem. Cell. Biol. **28**, 259–274

8 O'Sullivan, J., Unzeta, M., Healy, J., O'Sullivan, M. I., Davey, G. and Tipton, K. F. (2004) Semicarbazide-sensitive amine oxidases: enzymes with quite a lot to do. Neurotoxicology **25**, 305–315

9 Salminen, T. A., Smith, D. J., Jalkeanen, S. and Johnson, M. S. (1998) Structural model of the catalytic domain of an enzyme with cell adhesion activitity: human vascular adhesion protein-1 (HVAP-1) D4 domain is an amine oxidase. Prot. Eng. **11**, 1195–1204

10 Boosma, F., Bhaggoe, U. M., van der Houwen, A. M. B. and van der Meiracker, A. H. (2003) Plasma semicarbazide-sensitive amine oxidase in human (patho)physiology. Biochim. Biophys. Acta **1647**, 48–54

11 Pietrangeli, P. and Mondovì, B. (2004) Amine oxidases and tumors. Neurotoxicology **25**, 317–324

12 Shepard, E. M., Smith, J., Bradley, O. E., Kuchar, J. A., Sayre, M. L. and Dooley, D. M. (2002) Towards the development of selective amine oxidase inhibitors. Mechanism-based inhibition of six copper containing amine oxidases. Eur. J. Biochem. **269**, 3645–3658

13 Artico, M., Corelli, F., Massa, S., Stefanich, G., Avigliano, L., Befani, O., Marcozzi, G., Sabatini, S. and Mondovì, B. (1988) Inhibition of copper dependent amine oxidases by some hydrazides of pyrrol-1-ylbenzoic and pyrrol-1-ylacetic acids. J. Med. Chem. **31**, 802–806

14 Kinemuchi, H., Sugimoto, H., Obata, T., Satoh, N. and Ueda, S. (2004) Selective inhibitors of membrane-bound semicarbazide-sensitive amine oxidase (SSAO). Activity in mammalian tissues. Neurotoxicology **25**, 325–335

15 Bardsley, W. G., Ashford, J. S. and Hill, C. M. (1971) Synthesis and oxidation of ammonium alkyl-onium compounds by pig kidney diamine oxidase. Biochem. J. **122**, 557–567

16 Jeon, H. B., Sun, G. and Sayre, L. M. (2003) Inactivation of bovine plasma amine oxidase by 4-aryloxy-2-butynamines and related analogues. Biochim. Biophys. Acta **1647**, 343–354

17 Jeon, H. B. and Sayre, L. M. (2003) Highly potent propargylamine and allylamine inhibitors of bovine plasma amine oxidase. Biochem. Biophys. Res. Commun. **304**, 788–794

18 Banchelli, G., Buffoni, F., Elliot, J. and Callingham, B. A. (1990) A study of the biochemical pharmacology of 3,5-ethoxy-4-aminomethylpiridine (B24), a novel amine oxidase inhibitor with selectivity for tissue bound semicarbazide-sensitive amine oxidase enzymes. Neurochem. Int. **17**, 215–221

19 Lyles, G. A., Marshall, C. M. S., McDonald, I. A., Bey, P. and Palfreyman, M. G. (1987) Inhibition of rat aorta semicarbazide-sensitive amine oxidase by 2-phenyl-3-haloallylamines and related compounds. Biochem.Pharmacol. **36**, 2847–2853

20 Delikatny, E. J., Cooper, W. A., Sathasuvam-Nalayni, B. S. and Rideout, D. C. (2002) Nuclear magnetic resonance-visible lipids induced by cationic lipophilic chemotherapeutic agents are accompanied by increased lipid droplet formation and damaged mitochondria. Cancer Res. **62**, 1394–1400

21 Patel, J., Rideout, D., McCarthy, M. R., Calogeropoulou, T., Wadwa, K. S. and Oseroff, A. R. (1994) Antineoplastic activity, synergism and antagonism of triarylalkylphosphonium salts and their combinations. Anticancer Res. **14**, 21–28

22 Kanazawa, A., Ikeda, T. and Endo, T. (1994) Synthesis and antimicrobial activity of dimethyl and trimethyl-substituted phosphonium salts with alkyl chains of various lengths. Antimicrob. Agents Chemother. **38**, 945–952

23 Kanazawa, A., Ikeda, T. and Endo, T. (1995) A novel approach to mode of action of cationic biocides: pharmacological effect on antibacterial activity. J. Appl. Bacteriol. **78**, 55–60

24 Rotenberg, S. A., Calogeropoulou, T., Jaworski, J. S., Weinstein, I. B. and Rideout, D. (1991) A self-assembling protein kinase C inhibitor. Proc. Natl. Acad. Sci. U.S.A. **88**, 2490–2494

25 Molteni, V., Greenwald, J., Rhodes, D., Hwang, Y., Kwiatowski, W., Bushman, F. D., Siegel, J. S. and Choe, S. (2001) Identification of a small-molecule binding site at the dimer interface of HIV integrase catalytic domain. Acta Crystallogr. D Biol. Crystallogr. **57**, 536–544

26 Ono, A., Miyauchi, S., Depura, M., Asakura, T. and Kamo, N. (1994) Activation energy for permeation of phosphonium cations through phospholipid bilayer membrane. Biochemistry **33**, 4312–4318

27 Vianello, F., Di Paolo, M. L., Stevanato, R. and Rigo, A. (1992) Isolation of amine oxidase from bovine plasma by a two-step procedure. Protein Expression Purif. **3**, 362–367

28 Vianello, F., Di Paolo, M. L., Stevanato, R., Gasparini, R. and and Rigo, A. (1993) Purification and characterization of amine oxidase from soybean seedlings. Arch. Biochem. Biophys. **307**, 35–39

29 Di Paolo, M. L., Scarpa, M. and Rigo, A. (1994) A sensitive spectrophotometry-based method for the determination of the rate of hydrogen peroxide generation in biological systems. J. Biochem. Biophys. Methods **28**, 205–214

30 Oi, S., Inamasu, M. and Yasunobu, K. T. (1970) Mechanistic studies of beef plasma amine oxidase. Biochemistry **9**, 3378–3383

31 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254

32 Dixon, M. (1953) The determination of enzyme inhibition constants. Biochem. J. **55**, 170–171

33 Markley, J. L. (1973) High-resolution proton magnetic resonance studies of two trypsin inhibitors: soybean trypsin inhibitor (Kunitz) and ovomucoid (hen's egg white). Ann. N.Y. Acad. Sci. **222**, 347–373

34 Yu, P. H., Bruce, B. A. and Deng, Y. (2001) 2-Bromoethylamine as a potent selective suicide inhibitor for semicarbazide-sensitive amine oxidase. Biochem. Pharmacol. **61**, 741–748

35 Luhova, L., Slavik, L., Frebort, I., Sebela, M., Zajoncova, L. and Pecet, P. (1996) Comparison of kinetic properties between plant and fungal amine oxidases. J. Enzyme Inhib. **10**, 251–262

36 Di Paolo, M. L., Stevanato, R., Corazza, A., Vianello, F., Lunelli, L. and Scarpa, M. and Rigo A. (2003) Electrostatic compared with hydrophobic interactions between bovine serum amine oxidase and its substrates. Biochem. J. **371**, 549–556

37 Leidler, K. and Bunting, P. (1973) The steady state in enzyme kinetics. In The Chemical Kinetics of Enzyme Action (2nd edn.), pp. 68–113, Clarendon Press, Oxford

38 Di Paolo, M. L., Scarpa, M., Corazza, A., Stevanato, R. and Rigo, A. (2002) Binding of cations of group IA and IIA to bovine serum amine oxidase. Effect on the activity. Biophys. J. **83**, 2231–2239

39 Krupka, R. M. and Laidler, K. J. (1961) Molecular mechanism for hydrolytic enzyme action. I. Apparent non-competitive inhibition, with special reference to acetylcholinesterase. J. Am. Chem. Soc. **83**, 1445–1447

40 Shapiro, R. and Riordan, J. F. (1982) Inhibition of angiotensin converting enzyme: mechanism and substrate dependence. Biochemistry **23**, 5225–5233

41 Cho, U. S., Park, E. Y., Ding, M. S., Park, B. S., Kim, K. and Kim, K. H. (2003) Tight-binding inhibition by α-naphthoflavone of human cytochrome P450 1A2. Biochim. Biophys. Acta **1648**, 195–202

42 Atkins, P. W. (1986) Equilibrium electrochemistry: ions and electrodes. In Physical Chemistry (3rd edn.), pp. 242–243, Oxford University Press, Oxford
43 Leidler, K. and Bunting, P. (1973) General kinetic principles. In The Chemical Kinetics of Enzyme Action. (2nd edn), pp. 42–46, Clarendon Press, Oxford
44 Artico, M., Silvestri, R., Stefanich, G., Avigliano, L., Di Giulio, A., Maccarrone, M., Agostinelli, E., Mondovì, B. and Morpurgo, L. (1992) Aromatic hydrazides as specific inhibitors of bovine serum amine oxidase. Eur. J. Med. Chem. **27**, 219–228
45 Palcic, M. M. and Klinman, J. P. (1983) Isotopic probes yield microscopic constants: separation of binding energy from catalytic efficiency in the bovine plasma amine oxidase reaction. Biochemistry **22**, 5957–5966
46 Hartmann, C. and Klinmn, J. P. (1991) Structure–function studies of substrate oxidation by bovine serum amine oxidase: relationship to cofactor structure and mechanism. Biochemistry **30**, 4605–4611
47 Piekarska, A. E. and McPherson, G. A. (1997) Structure–activity relationship of quaternary ion antagonism of levcromakalim-induced relaxation in pig coronary artery. Eur. J. Pharmacol. **322**, 37–44
48 Min, J. J., Biswal, S., Deroose, C. and Gambhir, S. S. (2004) Tetraphenylphosphonium as a novel molecular probe for imaging tumors. J. Nucl. Med. **45**, 636–643
49 Holt, A., Sharman, D. F., Baker, G. B. and Palcic, M. M. (1997) A continuous spectrophotometric assay for monoamine oxidase and related enzymes in tissue homogenates. Anal. Biochem. **244**, 384–392

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